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(54) Title: METHODS OF PROMOTING THE SURVIVAL AND DIFFERENTIATION OF SUBCLASSES OF CHOLINERGIC AND SEROTONERGIC NEURONS USING FIBROBLAST GROWTH FACTOR-5

(57) Abstract

The present invention relates to methods of promoting the survival and differentiation of subclasses of cholinergic and serotonergic neurons using fibroblast growth factor-5 (FGF-5). FGF-5 promotes the differentiation of cholinergic septal neurons as well as serotonergic neurons of the raphe nucleus.

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METHODS OF PROMOTING THE SURVIVAL AND DIFFERENTIATION OF SUBCLASSES OF CHOLINERGIC AND SEROTONERGIC NEURONS USING FIBROBLAST GROWTH FACTOR-5

1. INTRODUCTION

The present invention relates to methods of promoting the survival and differentiation of subclasses of cholinergic and serotonergic neurons using fibroblast growth factor-5 (FGF-5). It is based, in part, on the discovery that FGF-5 promotes the differentiation of cholinergic septal neurons as well as serotonergic neurons of the raphe nucleus. FGF-5 may be used to promote the maintenance of hippocampal neural circuits.

2. BACKGROUND OF THE INVENTION

2.1. GROWTH FACTORS AND THE FIBROBLAST GROWTH FACTOR FAMILY

The differentiation of neurons is thought to reflect the interplay between genetic and epigenetic factors including growth factors and hormones. Different growth factors could act in concert or consecutively in a temporal sequence affecting the maturation and the maintenance of the neurons (Barde, 1989, Neuron 2:1525-1534). For example, it has been shown that early cerebellar granule neurons are responsive to BDNF whereas NT-3 might act on the more mature granule cell (Segal et al., 1992, Neuron 9:1041-1052). However, only BDNF and not NT-3 supports the survival of cultured cerebellar granule neurons from P2 (Segal et al., 1992, Neuron 9:1041-1052) and P7 rats. There is also an interaction between NGF and bFGF in neuronal development, as shown for rat striatal neuronal precursors in culture (Cattaneo and McKay, 1990, Nature 347:762-765). In addition, NGF (Hefti et al., 1985, Neuroscience 14:55-

68; Mobley et al., 1986, Mol. Brain Res. 1:53-62; Hatanaka et al., 1988, Dev. Brain Res. 39:85-95; Hartikka and Hefti, 1988, J. Neurosci. 8:2967-2985; Grothe et al., 1989, Neuroscience 3:649-661; Knüsel et al., 1990, J. Neurosci. 10:558-570), BDNF (Alderson et al., 1990, Neuron 5:297-306, Knüsel et al., 1991, Proc. Natl. Acad. Sci. USA 88:961-965) and bFGF (Grothe et al., 1989, Neuroscience 3:649-661) all have been shown to induce ChAT activity in cultured rat septal cholinergic neurons.

The fibroblast growth factor (FGF) family comprises seven different polypeptide factors with a great variety of biological effects on different cell types (for review see Burgess et al., 1989, Annu. Rev. Biochem. 58:575-606; Goldfarb, 1990, Cell Growth & Differentiation 1:439-445). The best characterized members of the FGF gene family, aFGF (acidic FGF) (Jaye et al, 1986, Science 233:541-545) and bFGF (basic FGF) (Abraham et al., 1986, Science 233:545-548) are expressed in different tissues and exhibit a mitogenic effect on many cultured cells including fibroblasts, endothelial cells, smooth muscle cells, myoblasts and astrocytes. Considerable amounts of aFGF and bFGF are present in the nervous system (Gospodarowicz et al., 1984, Proc. Natl. Acad. Sci. USA 81:6963-3967; Pettmann et al., 1985, FEBS Lett. 189:102-108; Emoto et al., 1989, Growth Factors 2:21-29; Eckenstein et al., 1991, J. Neurosci. 11:412-419; Gomez-Pinilla and Cotman, 1992, Mol. Brain Res. 606:79-86; Woodward et al., 1992, J. Neurosci. 12:142-152). bFGF has a widespread occurrence in the rat brain (Eckenstein et al., 1991, J. Neurosci. 11:412-419) and in the rat hippocampus it is localized mainly to neurons of the CA-2 region (Gomez-Pinilla and Cotman, 1992, Mol. Brain Res. 606:79-86; Woodward

et al., 1992, J. Neurosci. 12:142-152). Both aFGF and bFGF have been shown to have survival promoting activity on cultured peripheral and central neurons (for review see Walicke and Baird, 1988, Prog. Brain Res. 78:333-338). Thus, bFGF increases the survival of chick neurons isolated from ciliary ganglion and spinal cord (Unsicker et al., 1987, Proc. Natl. Acad. Sci. USA 84:5459-5463) and supports the survival of cultured rat hippocampal (Walicke et al., 1986, Proc. Natl. Acad. Sci. USA 83:3012-3016) and cortical neurons (Morrison et al., 1986, Proc. Natl. Acad Sci. USA 83:7537-7541). bFGF also enhances the differentiation of rat septal cholinergic neurons as manifested by an increase in choline acetyltransferase (ChAT) activity (Knüsel et al., 1990, J. Neurosci. 10:558-570; Hartikka et al., 1992, J. Neurosci. Res. 32:190-201). Local administration of bFGF has been reported to prevent degeneration of medial septum cholinergic neurons after fimbria fornix lesion in the rat (Anderson et al., 1988, Nature 332:360-361; Otto et al., 1989, J. Neurosci. Res. 22:83-91).

In contrast to the vast literature on the various biological effects of aFGF and bFGF, little is known about their mode of release from the producing cells. Both aFGF and bFGF lack a typical signal sequence, precluding their release via the classical secretory Endoplasmic Reticulum-Golgi pathway. It has been suggested that these factors might be released preferentially as a consequence of tissue damage (Burgess et al., 1989, Annu. Rev. Biochem. 58:575-606).

The protein originally termed FGF-3, but now designated FGF-5, was discovered as the gene product of an oncogene called ORF-2 (Goldfarb et al., PCT Publication WO 88/09378 dated December 1, 1988; Zhan

et al., 1987, Oncogene 1:369-376; Zhan et al., 1988, Mol. Cell. Biol. 8:3487-3495). The FGF-5 protein has 268 amino acids (corresponding to the second open reading frame or "ORF 2" as disclosed in Goldfarb et al., *supra*), the sequence of which is well-conserved across mammals. The murine homologue shows 84% overall sequence identity to the human (Hébert et al., 1990, Dev. Biol. 138:454-463). FGF-5, in contrast to aFGF and bFGF, has a hydrophobic leader sequence, typical of a secreted protein (Zhan, 1988, Mol. Cell. Biol. 8:3487-3495). Indeed, FGF-5 is secreted into the culture medium of transformed NIH 3T3 fibroblasts (Bates et al., 1991, Mol. Cell. Biol. 11:1840-1845). There is widespread expression of FGF-5 in different tissues during embryonic development of the mouse (Haub et al., 1991, Proc. Natl. Acad. Sci. USA 87:8022-8026) suggesting important functions for FGF-5 during this period (Hébert et al., 1990, Dev. Biol. 138:454-463). In addition, FGF-5 mRNA has been found to be present in the adult mouse brain and seems to be localized in central neurons as shown by *in situ* hybridization (Haub et al., 1990, Proc. Natl. Acad. Sci. USA 87:8022-8026). Gomez-Pinilla and Cotman, 1992, Mol. Brain Res. 606:79-86 reported on the presence of FGF-5 mRNA in the adult rat brain. According to their results using *in situ* hybridization, FGF-5 mRNA is localized mainly in neurons of hippocampus, olfactory bulb, olfactory cortex and entorhinal cortex.

It has recently been shown that FGF-5 mRNA is expressed in rat skeletal muscle both during embryonic development and in adulthood and that recombinant FGF-5 supports the survival of cultured chick motoneurons (Hughes et al., 1993, Neuron 10:369-377).

Molecular cloning of the receptors for the FGFs has demonstrated the existence of at least four different membrane-spanning tyrosine kinase receptors binding the various FGFs with different affinities (for review see Partanen et al., 1992, Prog. Growth Factor Res. 4:69-83). Some of these receptors are also present in the brain tissue (Reid et al., 1990, Proc. Natl. Acad. Sci. USA 87:1596-1600); for example the FGF receptor-1 (flg) (Lee et al., 1989, Science 245:57-60; Safran et al., 1990, Oncogene 5:635-645) is expressed in various regions of the adult rat brain (Wanaka et al., 1990, Neuron 5:267-281). However, the exact nature of the receptor mediating the actions of FGF-5 brain and in other systems is not known. Interestingly, flg receptors were not found on septal cholinergic neurons (Wanaka et al., 1990, Neuron 5:267-281).

2.2. THE HIPPOCAMPUS AND ITS CONNECTIONS

The hippocampus is an important part of the limbic system, a component of the central nervous system hypothesized to play a role in the formation of emotions and the expression of instinctive drives (see, for example, Duus, 1983, "Topical Diagnosis in Neurology," Georg Thieme Verlag, New York, pp. 279-285). The limbic system derives its name from limbus, the Latin word for margin, as it comprises the so-called "limbic lobe" at the margin of the cerebral cortex, as well as associated structures, such as the entorhinal and septal areas, indusium griseum, amygdaloid complex, and mammillary bodies.

The hippocampus is a primitive cortical derivative which is considered to be part of the limbic lobe, although it is actually invaginated within the temporal lobe (Carpenter, 1976, "Core Text

of Neuroanatomy", Second Edition, Williams & Wilkins Co., Baltimore, "Carpenter" p. 23). The shape of the hippocampus resembles a seahorse, hence its name.

The hippocampus projects impulses to different parts of the nervous system through complex pathways (Carpenter, p. 269), which connects the hippocampus with certain thalamic nuclei, the hypothalamus, the midbrain reticular formation, serotonergic neurons of the raphe nucleus (Lidov and Molliver, 1982, Brain Res. Bull. 8:389-430), and cholinergic neurons in the septal region.

Clinically, the hippocampus and its projections are the major sites of disease in disturbances of memory (Mohr, 1984, "Manual of Clinical Problems In Neurology", Mohr., ed., Little, Brown and Co., Boston, p. 27). So-called "amnestic states" may result from hippocampal injury caused by Alzheimer's disease, chronic alcoholism, lack of oxygen (which particularly damages Sommer's sector of the hippocampus) and toxins. Ischemic infarctions caused by lack of oxygen in the area of brain supplied blood by the posterior cerebral artery result in damage to the posterior two-thirds of the hippocampus, whereas infarctions in the territory supplied by the choroidal artery involve the anterior one-third.

NGF-mRNA (Ayer-LeLievre et al., 1988, Science 240:1339-1341; Whittemore et al., 1988, J. Neurosci. Res. 20:403-410; Bandtlow et al., 1990, J. Cell Biol. 111:1701-1711; Gall et al., 1991, Mol. Brain Res. 9:113-123) and BDNF-mRNA (Hofer et al., 1990, EMBO J. 9:2459-2464; Phillips et al., 1990, Science 250:290-294; Ernfors et al., 1990, Neuron 5:511-526; Wetmore et al., 1990, Exp. Neurol. 109:141-152; Dugich-Djordjevic et al., 1992, Neuron 8:1127-1138; Berzaghi et al., 1993, J. Neurosci. 13:3818-3826) are

present in the rat hippocampus, which is the target area of projecting cholinergic fibers from the septal region.

Citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to methods of promoting the survival and differentiation of subclasses of cholinergic and serotonergic neurons using FGF-5. The inventors have discovered that FGF-5 promotes the differentiation of cholinergic septal neurons as well as serotonergic neurons of the raphe nucleus, and that FGF-5 is expressed in the hippocampus in a manner associated with muscarinic receptor activation.

In particular embodiments, the present invention provides for methods of promoting the differentiation of septal cholinergic neurons or raphe serotonergic neurons, comprising exposing said neurons to effective concentrations of FGF-5.

In additional embodiments, the present invention provides for methods of increasing FGF-5 synthesis in the hippocampus via stimulation of muscarinic receptors, and, conversely, methods of decreasing FGF-5 synthesis in the hippocampus via inhibiting the activity of muscarinic receptors.

FGF-5 may be a target derived growth factor which sustains neurons which project to the hippocampus. Accordingly, the present invention also provides for methods of maintaining hippocampal neural circuits which comprise exposing the components of such circuits to effective concentrations of FGF-5.

3.1. ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
FGF	fibroblast growth factor
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
K-FGF	keratinocyte growth factor
FGF-5	fibroblast growth factor-5
mRNA	messenger RNA
NGF	nerve growth factor
NT-3	neurotrophin-3
PBS	phosphate buffered saline

4. DESCRIPTION OF THE FIGURES

FIGURE 1. FGF-5 expression in the rat hippocampus. *In situ* hybridization was performed on adult rat hippocampus as described in Methods. A single-stranded FGF-5 DNA probe was prepared either in the anti-sense (A) or sense (B) direction. Note the accumulation of specific grains in the rat hippocampus and cortex and the absence of grains in controls, B. DG, dentate gyrus; CA1, CA3, subregions of hippocampus.

FIGURE 2. Regulation of FGF-5 mRNA in the rat hippocampus.

FIGURE 2A. FGF-5 mRNA levels during postnatal development. Hippocampal RNA was extracted and analyzed as described in Methods. The amount of FGF-5 mRNA was normalized to the amount of β-actin present and expressed per wet weight of tissue. Values represent means ± SD, n=3-5 experiments.

FIGURE 2B. Effect of pilocarpine and scopolamine on FGF-5 mRNA. P7 rats were treated with pilocarpine for 6 hours alone or in combination

with scopolamine (see Methods). RNA was extracted as described above. Values represent means SD, n=3. C, control; P, pilocarpine; S, scopolamine; S+P, scopolamine and pilocarpine. *, P<0.05 for P versus C.

FIGURE 3. FGF-5 immunoreactivity in rat hippocampus.

FIGURE 3A. P10 rats were treated with pilocarpine for 6 hours and the hippocampi were processed for FGF-5 immunohistochemistry as described in Methods. Left, FGF-5 antiserum stain neurons in the dentate gyrus. Right, preadsorption of the antiserum with the FGF-5 peptide reduced the intensity of staining. Magnification, 200 times.

FIGURE 3B. Dentate gyrus of adult rat hippocampus after pilocarpine. Left, neuronal staining using the FGF-5 antiserum. Right, control without the first antibody. Magnification, 400 times.

FIGURE 4. FGF-5 increases ChAT activity in septal cultures.

FIGURE 4A. Effect of FGF-5 on NGF and bFGF induced ChAT activity. Septal cultures were treated with 10 ng of either NGF, bFGF and FGF-5 alone or with FGF-5 combined with NGF and bFGF. ChAT was determined as described in Methods. Values represent means ± SD, n=4.

FIGURE 4B. Effect of anti-NGF-antibodies on FGF-5 induced ChAT activity. 10 ng per ml of NGF and FGF-5 was added to the septal cultures in the absence or presence of 10 µg of anti-NGF-antibodies (clone 27/21). Values represent means ± SD, n=3.

FIGURE 5. Effect of growth factors on serotonin uptake by raphe neurons. Raphe serotonergic neurons were isolated from E14 rats (see Methods). 10 ng of various growth factors was

added to the cultures and serotonin uptake (5-HT) was determined after 7 days of incubation as described in Methods. Values represent means ± SD (n=4) and expressed as percentage of uptake in control cultures.

FIGURE 6. DNA sequence (SEQ ID NO:1) and encoded amino acid sequence (SEQ ID NO:2) of human FGF-5. The sequence shown is from Genbank, accession no. M37825; Haub et al., 1990, Proc. Natl. Acad. Sci. USA 87:8022-8026. (*See also*, PCT Publication No. WO 88/09378 dated December 1, 1988.)

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of promoting the survival and differentiation of subclasses of cholinergic and serotonergic neurons using FGF-5. The inventors have discovered that FGF-5 promotes the differentiation of cholinergic septal neurons as well as serotonergic neurons of the raphe nucleus, and that FGF-5 is expressed in the hippocampus in a manner associated with muscarinic receptor activation.

In particular embodiments, the present invention provides for methods of promoting the differentiation of septal cholinergic neurons or raphe serotonergic neurons, comprising exposing said neurons to effective amounts of FGF-5.

In additional embodiments, the present invention provides for methods of increasing FGF-5 synthesis in the hippocampus via stimulation of muscarinic receptors, and, conversely, methods of decreasing FGF-5 synthesis in the hippocampus via inhibiting the activity of muscarinic receptors.

FGF-5 may be a target derived growth factor which sustains neurons which project to the hippocampus. Accordingly, the present invention also provides for methods of maintaining hippocampal neural circuits which comprise exposing the components of such circuits to effective concentrations of FGF-5.

The term "FGF-5," as used herein, refers to a factor having a sequence as set forth in Figure 6, or an equivalent sequence obtained from other species (*see, e.g.*, Hébert et al., 1990, Dev. Biol. 138:454-463).

FGF-5 for use in the present invention may be purified from natural sources, chemically synthesized, or, preferably, produced by recombinant methods. The FGF-5 protein for use in the invention is preferably substantially purified. Purification can be carried out by standard methods known in the art. For example, recombinant FGF-5 can be purified from the soluble fraction of *E. coli* lysates (*see, e.g.*, Goldfarb, PCT Publication No. WO 88/09378 dated December 1, 1988).

As detailed in the examples section *infra*, primary cultures from different regions of the embryonic rat brain were established, and recombinant FGF-5 was found to enhance the differentiation of cultured septal cholinergic neurons as measured by increased ChAT activity. The FGF-5-mediated induction of ChAT was smaller than that elicited by NGF or BDNF, but was additive to that of NGF and was not inhibited by anti-NGF-antibodies. FGF-5 also promoted the differentiation of rat serotonergic neurons, isolated from developing raphe nuclei, as judged by an increase in serotonin uptake. BDNF and NT-3 but not NGF also elevated serotonin uptake by cultured raphe neurons,

but the effects were smaller than those evoked by FGF-5.

In additional examples, *in situ* hybridization experiments revealed that FGF-5 mRNA is present in neurons of the rat hippocampus. The levels of FGF-5 mRNA were observed to increase during early postnatal development and to be up-regulated by pilocarpine, a muscarinic receptor agonist. Immunohistochemical studies demonstrated that FGF-5 protein is also present in the rat hippocampus, mainly in the dentate gyrus and in the CA3 region. Due to the secretory nature of the FGF-5 protein, FGF-5 is probably also released in the rat hippocampus, which is the target region of septal cholinergic and raphe serotonergic neurons.

For clarity, and not by way of limitation, the detailed description of the invention is divided into the following subsections.

(1) the use of FGF-5 to promote the differentiation of septal cholinergic neurons;

(2) the use of FGF-5 to promote the differentiation of raphe serotonergic neurons;

(3) methods of altering the expression of FGF-5 by the hippocampus;

(4) methods of promoting the integrity of hippocampal neural circuits;

(5) pharmaceutical compositions and methods of administration; and

(6) functional derivatives of FGF-5.

5.1. THE USE OF FIBROBLAST GROWTH FACTOR-5 TO PROMOTE THE DIFFERENTIATION OF SEPTAL CHOLINERGIC NEURONS

The present invention provides for methods of promoting differentiation of septal cholinergic neurons comprising exposing said neurons to an effective concentration of FGF-5.

"Promoting differentiation", as used herein, refers to maintenance or induction of the cholinergic phenotype. For example, and not by way of limitation, "promoting differentiation" of a septal cholinergic neuron may result in a maintenance of, or increase in, the level of choline acetyl transferase (ChAT) activity. The ability of the present invention to promote the differentiation of septal cholinergic neurons is exemplified in § 6.2.4, *infra*.

The septal cholinergic neurons may be exposed to FGF-5 either *in vitro* or *in vivo*. Further, such neurons may be of either a human or a non-human host, and are preferably mammalian, including but not limited to primates such as chimpanzees and baboons, as well as cows, horses, dogs, pigs, rabbits, mice and rats. *In vivo* exposure of septal cholinergic neurons to FGF-5 may be performed in human or non-human subjects. *In vitro* cultures of septal cholinergic neurons are established by methods known in the art, *e.g.*, as described in Section 6.1.3, *infra*. The extent of differentiation of cholinergic neurons is preferably assayed by measuring ChAT activity, *e.g.*, as described in Section 6.1.4, *infra*, but can also be assayed by acetyl cholinesterase histochemistry, or other methods known in the art.

The "effective amount," effective to promote differentiation of septal cholinergic neurons, may be determined by constructing dose response curves using standard methods which are known to the skilled

artisan. Such determinations may preferably be initially performed *in vitro*, followed by *in vivo* experiments. *In vitro* cultures of septal cholinergic neurons, absent neurotrophic factors, would be expected to gradually lose their differentiated cholinergic phenotype. Therefore, the ability of a particular concentration of FGF-5 to stabilize or increase ChAT activity in such cultures would be expected to be effective in promoting differentiation of septal cholinergic neurons *in vivo* in subjects suffering from a disorder which causes impairment, de-differentiation, or death of such neurons. Because, as discussed in § 6.2.4, *infra*, 3 nanograms (ng) per milliliter (ml) of FGF-5 was observed to increase ChAT activity but 50 ng/ml increased ChAT activity only slightly more, in non-limiting specific embodiments of the invention an effective concentration of FGF-5 is between about 1 and 75 ng/ml, and preferably is between about 10 and 50 ng/ml. A concentration of FGF-5 may also be considered effective if it increases ChAT activity in septal cholinergic neurons by at least about 150 percent.

The fact that the effects of FGF-5 on septal cholinergic neurons was additive to the effects of NGF (*see* examples *infra*) indicates that FGF-5 operates on a different receptor(s) than the receptor(s) utilized by members of the BDNF/NGF/NT-3/NT-4 neurotrophin family. Accordingly, in particular embodiments of the invention, FGF-5 together with a member of the neurotrophin family (including, but not limited to, BDNF, NGF, NT-3 or NT-4) may be used to promote differentiation of septal cholinergic neurons. An effective amount of such neurotrophin may be defined as that amount which increases ChAT activity in a

sample of septal cholinergic neurons by at least 150-200 percent, and, in nonlimiting specific embodiments, may be a concentration between about 1-50 ng/ml, and may be most preferably between about 10-20 ng/ml. In further embodiments, an effective concentration of a mixture of FGF-5 and neurotrophin together may achieve an increase in ChAT activity of at least 150 percent.

FGF-5, alone or in conjunction with neurotrophin, may be used to promote the differentiation of septal cholinergic neurons in culture. Such cultures may be used to assay aspects of the biology of such neurons outside of their response to FGF-5, *e.g.*, to assay the effects of potential therapeutics, to assay the toxicity of compounds upon such neurons, to assay the effect of different compounds on neuronal differentiation and neuronal gene expression, etc., or may be used for therapeutic purposes, *e.g.*, in the preparation of cells for transplantation. For example, and not by way of limitation, such cells could be genetically engineered to contain a gene of interest while in culture, and then transplanted into a host organism.

In vivo, FGF-5, alone or in conjunction with a neurotrophin, may be used to treat neurological disorders which involve septal cholinergic neurons, including conditions where septal cholinergic neurons are damaged by trauma, infarction, infection, malignancy, or toxin, as well as conditions where such neurons are functionally impaired, e.g., epilepsy. For example, such conditions may include disorders of memory or learning, including Alzheimer's disease or Downs syndrome.

5.2. THE USE OF FIBROBLAST GROWTH FACTOR-5 TO PROMOTE THE DIFFERENTIATION OF RAPHE SEROTONERGIC NEURONS

The present invention provides for methods of promoting differentiation of raphe serotonergic neurons comprising exposing said neurons to an effective concentration of FGF-5.

"Promoting differentiation", as used herein, refers to maintenance or induction of the serotonergic phenotype. For example, and not by way of limitation, "promoting differentiation" of a raphe serotonergic neuron may result in a maintenance of, or increase in, the level of serotonin uptake. The ability of the present invention to promote the differentiation of serotonergic raphe neurons is exemplified in § 6.2.5. *infra*.

The raphe serotonergic neurons may be exposed to FGF-5 either *in vitro* or *in vivo*. Further, such neurons may derive from either a human or a non-human host. *In vivo* exposure of raphe serotonergic neurons to FGF-5 may be performed in human or non-human subjects, preferably mammalian, including but not limited to primates such as chimpanzees and baboons, as well as cows, horses, dogs, pigs, rabbits, mice and rats. *In vitro* cultures of raphe serotonergic neurons are established by methods known in the art, *e.g.*, as described in Section 6.1.3, *infra*. The extent of differentiation of serotonergic neurons is assayed by measuring serotonin uptake, *e.g.*, as described in Section 6.1.5, *infra*, measuring serotonin levels (e.g., by immunohistochemistry or fluorometric assay), by determination of tryptophan hydroxylase (the enzyme which synthesizes serotonin) (*e.g.*, by activity assay or immunohistochemistry), or by other methods known in the art.

The "effective amount," effective to promote differentiation of raphe serotonergic neurons may be determined by constructing dose response curves using standard methods which are known to the skilled artisan. Such determinations may preferably be initially performed *in vitro*, followed by *in vivo* experiments. *In vitro* cultures of raphe serotonergic neurons, absent neurotrophic factors, would be expected to gradually lose their differentiated serotonergic phenotype. Therefore, the ability of a particular concentration of FGF-5 to stabilize or increase serotonin uptake in such cultures would be expected to be effective in promoting differentiation of raphe serotonergic neurons *in vivo* in subjects suffering from a disorder which causes impairment, de-differentiation, or death of such neurons. Because, as discussed in § 6.2.5, *infra*, 10 ng/ml of FGF-5 was found to increase the amount of serotonin uptake by about 80 percent, in non-limiting specific embodiments of the invention, an effective amount of FGF-5 is a concentration between about 1 and 100 ng/ml and preferably between about 10 and 50 ng/ml. A concentration of FGF-5 may also be considered effective if it increases serotonin uptake by a sample of serotonergic neurons by at least about 50 percent.

In further embodiments of the invention, FGF-5 may be combined with a member of the neurotrophin family (see *supra*), preferably BDNF or NT-3, to promote differentiation of raphe serotonergic neurons. An effective concentration of such neurotrophin may be defined as that amount which increases serotonin uptake by at least 10 percent, and, in nonlimiting specific embodiments, may be between about 1-50 ng/ml and may be most preferably between about 10-20 ng/ml. In further embodiments, an effective concentration of

a mixture of FGF-5 and neurotrophin together may achieve an increase in serotonin uptake of at least 50 percent.

For *in vivo* embodiments, FGF-5 (and neurotrophin, in certain embodiments), may be administered by any method known in the art including, but not limited to, local instillation, intraventricular catheter, intrathecal administration, implant, or by subcutaneous, intramuscular, intravenous, intraarterial, intraperitoneal, intranasal, or aerosol inhalation routes.

FGF-5, alone or in conjunction with neurotrophin, may be used to promote the differentiation of raphe serotonergic neurons in culture. Such cultures may be used to study aspects of the biology of such neurons outside of their response to FGF-5, *e.g.*, to assay the effects of potential therapeutics, to assay the toxicity of compounds upon such neurons, to assay the effect of different compounds on neuron differentiation and neuronal gene expression, etc., or may be used for therapeutic purposes in the preparation of cells for transplantation. For example, and not by way of limitation, such cells could be genetically engineered to contain a gene of interest while in culture, and then transplanted into a host organism.

In vivo, FGF-5, alone or in conjunction with a neurotrophin, may be used to treat neurological disorders which involve raphe serotonergic neurons, including conditions where raphe neurons are damaged by trauma, infarction, infection, malignancy, or toxin, as well as conditions where such neurons are functionally impaired, e.g., epilepsy.

5.3. METHODS OF ALTERING THE EXPRESSION OF FIBROBLAST GROWTH FACTOR-5 BY THE HIPPOCAMPUS

In further embodiments, the present invention provides for methods of increasing expression of FGF-5 by the hippocampus of a subject comprising administering an effective dose of a muscarinic receptor agonist to the subject.

Increased expression of FGF-5 refers to increased levels of FGF-5 mRNA and/or protein.

Muscarinic receptor agonists that may be used according to the invention include, but are not limited to pilocarpine, oxotremorine, carbachol, and betanecol.

An effective concentration of muscarinic receptor agonist is a concentration which increases the levels of FGF-5 mRNA in hippocampal cells by at least about 50%. In a specific nonlimiting embodiment of the invention where the agonist is pilocarpine, a dose of between about 100 mg/kg and 400 mg/kg, preferably 300-350 mg/kg, administered intraperitoneally, may be used for rodents.

The foregoing methods may be practiced *in vitro* or *in vivo*, in human or non-human subjects, preferably mammalian, including but not limited to primates such as chimpanzees and baboons, as well as cows, horses, dogs, pigs, rabbits, mice, and rats. The agonists may be administered by any appropriate manner known in the art, including but not limited to local instillation, intraventricular catheter, intrathecal administration, implant, or by subcutaneous, intramuscular, intravenous, intraarterial, intraperitoneal, intraocular, intranasal, or aerosol inhalation routes.

The utility of such methods *in vivo* includes therapy of lesions of neural circuits that include the hippocampus. As discussed more fully in the following

section, FGF-5 may be a target-derived neurotrophic factor, synthesized in the hippocampus, which maintains the viability and/or differentiation of neurons that project their processes to contact the hippocampus. By increasing FGF-5 expression by the hippocampus, the maintenance of neural circuits that include the hippocampus may be promoted.

Conversely, muscarinic receptor antagonists may be used to decrease expression of FGF-5 in the hippocampus.

5.4. METHODS OF PROMOTING THE INTEGRITY OF HIPPOCAMPAL NEURAL CIRCUITS

As described in § 6, *infra*, FGF-5 has been observed to promote the differentiation of septal cholinergic neurons as well as raphe serotonergic neurons. We have also observed that FGF-5 is expressed in hippocampal tissue. The hippocampus is a target for neural projections of both septal cholinergic neurons and raphe serotonergic neurons. The present invention provides for methods of promoting or maintaining neural circuits that include the hippocampus which comprise exposing neurons that are elements of such circuits to an effective concentration of FGF-5. Such neural circuits include connections between the hippocampus and the thalamus, hypothalamus, the midbrain reticular formation, serotonergic neurons of the raphe nucleus, cholinergic neurons in the septal region, and any other neuron of the central nervous system which has a fiber process that projects to the hippocampus.

Such an effective concentration of FGF-5 is the concentration of FGF-5 which promotes the survival and/or differentiation of a neuron that projects to the hippocampus. In particular, nonlimiting embodiments of the invention, the concentration of

FGF-5 may be between about 1 ng/ml and 100 ng/ml, and preferably between about 10 ng/ml and 50 ng/ml.

The FGF-5 may be administered to a human or non-human subject in need of such treatment by any method known in the art, including but not limited to those set forth in § 5.1, *supra*.

The utility of such methods includes the sustenance of neural circuits that include the hippocampus following an event that damages the hippocampus. For example, if a subject becomes anoxic, the hippocampus of the subject may be damaged in such a manner that the hippocampus at least temporarily is unable to synthesize sufficient FGF-5 to adequately sustain neurons in other structures to which it is connected. If such connections are interrupted, not only does the subject lose the association between subsystems of the brain, but the hippocampus itself is destroyed as well as its associated structures. Supplies of factors delivered to the hippocampus by projecting nerve fibers may be terminated so that the damaged hippocampus may be less likely to recover than if such factors were available. By supporting the survival of neurons that project to the hippocampus, the survival of the hippocampus itself may be promoted.

Such methods may be used to promote the integrity of hippocampal neural circuits when the hippocampus has been damaged by trauma, infarction, infection, malignancy, toxin, an inborn error of metabolism, or a degenerative disorder. In a specific embodiment, such methods may be used in the treatment of Alzheimer's disease.

5.5. PHARMACEUTICAL COMPOSITIONS AND METHODS OF ADMINISTRATION

For *in vivo* embodiments, FGF-5 may be administered by any method known in the art, including, but not limited to, local instillation, encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a FGF-5 nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, intraarterial, subcutaneous, intranasal, oral, or aerosol inhalation routes. The compound may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a

suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of FGF-5, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for

example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The FGF-5 can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of FGF-5 which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body

weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

5.6. FUNCTIONAL DERIVATIVES OF FGF-5

The present invention also envisions the use of functional derivatives of FGF-5, in the methods and compositions disclosed above using FGF-5. By such a functional derivative is meant a derivative which retains the desired biological activity of FGF-5, *e.g.*, promotion of differentiation of raphe serotonergic neurons, promotion of differentiation of septal cholinergic neurons, etc., as the case may be, relevant to the desired use of the derivative. Derivatives of FGF-5 can be tested for the desired biological activity by procedures known in the art, including but not limited to the assays described herein.

In particular, FGF-5 derivatives can be made by altering FGF-5 sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an FGF-5 gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of FGF-5 genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the FGF-5 derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of an FGF-5 protein including altered sequences in which functionally

equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The FGF-5 derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned FGF-5 gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1989, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of FGF-5, care should be taken to ensure that the modified gene remains within the same translational reading frame as FGF-5, uninterrupted by translational stop signals, in the

gene region where the desired FGF-5 activity is encoded.

Additionally, the FGF-5-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), etc.

Manipulations of the FGF-5 sequence may also be made at the protein level. Included within the scope of the invention are FGF-5 protein fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of FGF-5 can be chemically synthesized. For example, a peptide corresponding to a portion of a FGF-5 protein which comprises the desired domain, or which mediates the desired aggregation activity *in vitro*, or binding

to a receptor, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the FGF-5 sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, and Nα-methyl amino acids.

In a specific embodiment, the FGF-5 derivative is a chimeric, or fusion, protein comprising a FGF-5 protein or fragment thereof fused via a peptide bond at its amino- and/or carboxy-terminus to an amino acid sequence of a protein other than FGF-5. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising an FGF-5-coding sequence joined in-frame to a non-FGF-5 coding sequence). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

6. EXAMPLE: FIBROBLAST GROWTH FACTOR-5 PROMOTES DIFFERENTIATION OF CULTURED RAT SEPTAL CHOLINERGIC AND RAPHE SEROTONERGIC NEURONS AND COMPARISON WITH THE EFFECTS OF NEUROTROPHINS

6.1. MATERIALS AND METHODS

6.1.1. MATERIALS

Recombinant human FGF-5 was produced in E. Coli and was a kind gift from Mitchell Goldfarb, Columbia University. NGF was prepared from male mouse submandibular glans and recombinant mouse BDNF and NT-3 were produced in the vaccinia system (Götz et al., 1992, Eur. J. Biochem. 204:745-749). Bovine bFGF was from Boehringer Mannheim, FRG. Monoclonal anti-NGF-antibodies were produced from hybridoma cells (clone 27/21) (Korshing and Thoenen, 1983, Proc. Natl. Acad. Sci. USA 80:3513-3516). Pargyline, amitriptyline and paroxetine were from Research Biochemicals Inc. Empigen was purchased from Chemische Fabrik Schweizerhall, Basel, Switzerland. Ultima Gold was from Packard Instruments. The ABC Elite kit (Vector Lab) was from Camon, Wiesbaden, FRG. All other reagents were obtained from Sigma.

6.1.2. ANIMAL TREATMENT

Male Wistar rats were used. The date of birth was defined as PO. Pilocarpine was administered intraperitoneally at 150 or 340 mg/kg, and methylscopolamine (1 mg/kg) was given to some animals subcutaneously, in order to avoid peripheral muscarinic side effects, 30 minutes prior to pilocarpine (Berzaghi, 1993, J. Neurosci. 13:3818-3826). Pretreatment of other animals with scopolamine to block the brain muscarinic receptors was performed 45 minutes before pilocarpine. After various times of treatment hippocampus was dissected, and immediately

frozen for analysis of RNA. All animal experiments were conducted according to rules stipulated by the government of Bavaria.

6.1.3. CELL CULTURES

Brains were removed from rat fetuses at embryonic day 17 (E17), and the septal regions containing the cholinergic neurons were carefully dissected under a stereomicroscope (Hartikka and Hefti, 1988, J. Neurosci. 8:2967-2985). The tissue was subsequently incubated for 20 minutes at 37°C in phosphate buffered saline (PBS) containing 10 mM glucose, 1 mM albumin, 6 µg/ml DNase and 12 U/ml papain but no magnesium or calcium ions. The tissue pieces were gently triturated with a plastic pipette, and the cells were collected by low speed centrifugation (900xg for 5 minutes) and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal calf serum. The cells were plated on poly-DL-ornithine precoated Costar 24 well dishes at a density of 0.4 x 0^6 cells per dish. Three hours after plating, the medium was changed to a serum-free one prepared as previously described for hippocampal and cerebellar neurons (Zafra et al., 1990, EMBO J. 9:3545-3550). Cultures were maintained at 37°C in a 95% air/5% CO_2 humidified atmosphere. The medium was changed after five days of incubation and the various growth factors and neurotrophins were added at the concentrations indicated. Following another five days of incubation ChAT activity in the cultures were determined as described below.

E14 old rat fetuses (crown-rump length 10-11 mm) were used to prepare serotonergic neurons. The rostral rhombencephalon containing the cell groups B4 to B9, *i.e.* nucleus raphe medialis and nucleus raphe

pontis (Lidov and Molliver, 1982, Brain Res. Bull. 8:389-430; Wallace et al., 1983, Brain Res. Bull. 10:459-479) were dissected and carefully separated from the noradrenergic neurons of the locus coeruleus (Foguet et al., 1993, EMBO J. 12(3):903-910). After dissection, tissue pieces were digested with papain as described above and carefully dissociated with a Pasteur pipette. The neurons (0.25-0.35x10^6 cells) were plated onto Costar 24 well culture dishes pre-coated with poly-DL-ornithine and incubated in the serum-free medium described above. Various growth factors and neurotrophins were added to the cultures at the beginning of the incubation.

6.1.4. DETERMINATION OF ChAT ACTIVITY

The septal cultures were washed three times with PBS and then homogenized in 0.5 ml of 50 mM Tris-HCl (pH 6.0) with 0.3% Triton X-100. 50 μl of the homogenate was taken for the determination of choline acetyl transferase (ChAT) activity by a modification of the method of Fonnum, 1975, J. Neurochem. 24:407-409. The reaction mixture contained 0.25 mM acetyl co-enzyme A (acetyl-CoA), 0.5 μC ^{3}H acetyl-CoA (specific activity 4.4 Ci/mmol), 10 mM choline chloride, 300 mM NaCl and 0.2 mM physostigmine in 50 mM sodium phosphate buffer, pH 7.4. Following incubation at 37°C for 30 minutes the reaction was stopped by adding 2 ml of 10 mM sodium phosphate buffer and transferred to a scintillation vial. The amount of labeled acetylcholine formed was counted using a toluene-based scintillation cocktail and sodium tetraphenylboron (Fonnum, 1975, J. Neurochem. 24:407-409). Control incubations were performed using buffer only or boiled cell lysates. ChAT activity (cpm in the assay minus buffer blanks) present in the

cultures were expressed per well and per mg protein which was measured using a kit (BioRad) and serum albumin as a standard. Statistical analyses were performed using the Student's t-test.

6.1.5. SEROTONIN UPTAKE

Cultures containing raphe serotonergic neurons were washed three times with 25% Hanks balanced salt solution (HBSS) and pre-incubated for 10 minutes in HBSS containing 6 mg/ml glucose, 1 mM ascorbic acid and 0.1 mM pargyline (a monoamine oxidase inhibitor). Radiolabeled serotonin (^{3}H serotonin; 20-30 Ci/mmol) was then added to a final concentration of 50 nM. The incubation was carried out for 20 minutes at room temperature and the reaction was stopped by removing the medium following three rapid washes with cold HBSS. Cultures were then extracted twice with 0.3 ml of 1% Empigen, 3.5 ml Ultima Gold was added per scintillation vial and the amount of radioactivity was determined. The nonspecific uptake of serotonin was determined using the drugs paroxetine (1 μM) and amitriptyline (10 μM) (Buus, 1978, Eur. J. Pharmacol. 47:351-358).

6.1.6. NORTHERN BLOTS

Total cellular RNA was isolated from different regions of rat brain by the method of Chomczynski and Sacchi, (1987, Anal. Biochem. 162:156-159). Purified RNA (about 20 μg) was glyoxylated, electrophoresed through a 1.4% agarose gel and transferred to a nylon membrane (Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351). The filters were hybridized overnight using 50% formamide as described in Castren et al. (1992, Proc. Natl. Acad. Sci. USA 89:9444-9448) in the presence of a ^{32}P-labeled cRNA probe for mouse

FGF-5 (specific activity 10^9 cpm/μg). The probe was made by run-on transcription from a Bluescript vector containing a 2.2 kb mouse FGF-5 cDNA (Haub et al., 1991, Proc. Natl. Acad. Sci. USA 87:8022-8026). After washing, the filters were exposed to x-ray films and subsequently rehybridized with a cRNA probe for β-actin (Lindholm et al., 1988, J. Biol. Chem. 263:16348-16351) as a control for the amount of RNA present in each lane.

6.1.7. *In Situ* HYBRIDIZATION

In situ hybridization was performed in 12-μm-thick frozen sections, which were post-fixed in a 4% buffered paraformaldehyde, treated with acetic anhydride, and hybridized overnight at 42°C in a buffer containing 50% formamide, 4 X SSC, 50 mM phosphate buffer (pH 7.0), 1% lauryl sarkosine 1 x Denhardt's solution, 0.5 mg of denatured salmon sperm DNA per ml, 10% Dextran sulphate, and 100 mM dithiotreitol. The probe was a single-stranded ^{35}S-labeled cDNA probe transcribed from FGF-5 sense cRNA with reverse transcriptase and random priming to a specific activity of 2-3x10^9 cpm/μg (Castren et al., 1992, Proc. Natl. Acad. Sci. USA 89:9444-9448). The sections were washed under increasing stringency up to 0.5 X SSC at 60°C, dehydrated and exposed to Hyperfilm β-Max for 14 days. Control sections were hybridized with a corresponding sense probe and showed no specific hybridization.

6.1.8. IMMUNOHISTOCHEMISTRY

An antibody for FGF-5 against the C-terminal region, Phe^{217} to Lys^{234}, was raised in New Zealand rabbits as described earlier (Hughes et al., 1993, Neuron 10:369-377). The antiserum, previously shown

to immunoprecipitate recombinant FGF-5 (Hughes et al., 1993, Neuron 10:369-377), was used for staining brain sections. In brief, rats were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). After post-fixation in the same solution and washing in increasing concentrations of sucrose the brains were frozen and 12 μm-thick sections were cut on a freezing microtome and mounted on glass slides (Lindholm et al., 1992, J. Cell Biol. 117:395-400). The sections were first incubated for 30 minutes with 10% normal goat serum in PBS and then for two days with the FGF-5 antiserum (diluted 1 to 100) in 0.1% Triton X-100 in PBS. After washing in PBS, the sections were incubated for 1 hour with the biotinylated second antibody and the immunoreaction product was visualized using the avidin-biotin method and the Vectastatin kit.

To verify the specificity of the observed immunostaining, control experiments were performed with FGF-5 antiserum immunoprecipitated prior to use with either the peptide antigen or a synthetic peptide derived from the human K-FGF sequence (Pro^{61}-Leu^{79}, Delli et al., 1987, Cell 50:729-737). Following HPLC purification as described (Hughes et al., 1993, Neuron 10:369-377) 0.5 μmol of each peptide was allowed to react with activated CNBr-derivatized Sepharose beads (50 mg per peptide) in coupling buffer (0.1 M NaHCO, 0.5 M NaCl, pH 8.3) for 2 hours at room temperature. The reactions were blocked by the addition of 0.2 M glycine, pH 8.0 for 2 hours at room temperature and the resultant Sepharose-peptide conjugates washed extensively prior to addition of FGF-5 antiserum (170 μl in 4350 μl PBS). After incubation (1 hour at 4°C) the beads were separated by centrifugation and three

supernatants were used to stain brain sections as described above.

6.2. RESULTS

6.2.1. DEVELOPMENTAL CHANGES IN FGF-5 mRNA LEVELS IN POSTNATAL RAT HIPPOCAMPUS

FGF-5 mRNA has previously been shown to be present in low amounts in adult mouse brain (Haub et al., 1991, Proc. Natl. Acad. Sci. USA 87:8022-8026). In order to study whether FGF-5 is also expressed in rat brain, we employed *in situ* hybridization techniques using a specific single-stranded DNA probe. As shown in Fig. 1, FGF-5 is expressed in the adult rat hippocampus mainly in the dentate gyrus and in the CA3 region. The pattern of hybridization led us to conclude that FGF-5 in the rat hippocampus is expressed mainly by neurons (Fig. 1). There were also scattered cells positive for FGF-5 in other parts of the brain outside the hippocampus, *e.g.* in the cortex (Fig. 1) but the cell types expressing FGF-5 in these areas could not be conclusively identified. As shown in Fig. 2A the levels of FGF-5 mRNA increases substantially in the rat hippocampus during the first three postnatal weeks reaching a maximum of P18 rats. A similar time-course has earlier been described for NGF mRNA in the developing rat hippocampus (Auburger et al., 1988, Dev. Biol. 120:322-328).

6.2.2. HIPPOCAMPAL FGF-5 mRNA IS REGULATED BY ACTIVATION OF MUSCARINIC RECEPTORS

Fig. 2B shows that pilocarpine, a muscarinic receptor agonist increased (70%) the levels of FGF-5 mRNA in P7 rat hippocampus. This effect seemed to be specific since scopolamine, a muscarinic receptor antagonist, inhibited the increase in FGF-5 mRNA by pilocarpine. Interestingly, scopolamine alone reduced

the basal levels of hippocampal FGF-5 mRNA in these rats (Fig. 2B) strongly suggesting that FGF-5 mRNA expression in the rat hippocampus is regulated by the activation of muscarinic receptors.

6.2.3. PRESENCE OF FGF-5 IMMUNOREACTIVITY IN THE RAT HIPPOCAMPUS

In order to study whether FGF-5 protein is also present in rat hippocampus, we employed an anti-peptide antiserum to FGF-5, for immunohistochemical analysis (Hughes et al., 1993, Neuron 10:369-377). Hippocampal neurons were immunopositive in the granular cell layer and the hilus of the dentate gyrus (Fig. 3A) and also in the CA3 region. The possible presence of FGF-5 in glial cell as shown by staining of fiber elements (Fig. 3) cannot be excluded. Moreover, the intensity of staining was increased by pilocarpine. Some neurons seemed to be more intensely stained than others as is evident in the larger magnification of the dentate gyrus area (Fig. 3B). The pattern of staining of FGF-5 protein in rat hippocampus using this antibody corresponds closely to that seen for FGF-5 mRNA using *in situ* hybridization (Fig. 1). The specificity of FGF-5 immunohistochemistry was determined by performing control experiments with FGF-5 antiserum which had been immunoprecipitated prior to use. The intensity of staining was reduced if the antiserum was immunoprecipitated with the peptide antigen coupled to Sepharose (Fig. 3A). In contrast, staining was not affected if the FGF-5 antiserum was immunoprecipitated with a peptide of similar length derived from the sequence of another member of the FGF family, K-FGF.

6.2.4. FGF-5 INCREASES ChAT ACTIVITY IN CULTURED SEPTAL CHOLINERGIC NEURONS

Table 1 shows that 3 ng/ml of FGF-5 increased the ChAT activity of cultured septal neurons by 60%, whereas 50 ng/ml of FGF-5 elevated ChAT only slightly more. Due to the limited availability of recombinant FGF-5, it was not possible to study the effects of higher concentrations of FGF-5 on ChAT activity. Moreover, heparin, which is known to potentiate the effects of different FGFs (Klagsbrun and Baird, 1991, Cell 67:229-231) could not be used here as many neurons detached from the culture dishes after longer incubation with heparin.

bFGF, which has previously been shown to act on septal cholinergic neurons (Grothe et al., 1989, Neuroscience 3:649-661; Knüsel et al., 1990, J. Neurosci. 10:558-570) increased ChAT to about the same extent as FGF-5. Moreover, the addition of NGF and BDNF (20 ng/ml) increased the ChAT activity about 180% in these cultures (Table 1), a result consistent with studies described in Alderson et al., 1990, Neuron 5:297-306 and Knüsel et al., 1991, Proc. Natl. Acad. Sci. USA 88:961-965.

TABLE 1. EFFECT OF GROWTH FACTORS ON ChAT ACTIVITY*

Concentration (ng/ml)	ChAT activity (pmol/min/mg protein, % of control)	
Control	440 ± 29	-
NGF (20 ng)	1214 ± 126	275
BDNF (20 ng)	1408 ± 60	320
FGF-5 (3 ng)	704 ± 15	160
FGF-5 (10 ng)	721 ± 49	163
FGF-5 (50 ng)	823 ± 42	190
bFGF (10 ng)	662 ± 81	140
bFGF (50 ng)	839 ± 49	190

Septal cultures were established from E17 rats and treated for 5 days with various concentrations of growth factors (see Methods). ChAT activity was determined by the method of Fonnum, 1975, J. Neurochem. 24:407-409 and the results expressed as pmol acetylcholine formed per min and mg protein. Values represent the mean ± SD, n=6.

The effect of FGF-5 on the ChAT activity was additive to that of NGF (Fig. 4A), and anti-NGF antibodies did not reduce the FGF-5-mediated increase in ChAT activity in the septal cultures (Fig. 4B). However, the effects of FGF-5 and bFGF were not additive (Fig. 4A) suggesting the involvement of the same type of FGF receptor(s).

6.2.5. FGF-5 PROMOTES DIFFERENTIATION OF CULTURED RAPHE SEROTONERGIC NEURONS

To evaluate whether the action of FGF-5 is restricted to septal cholinergic neurons, we studied

its effects on cultured serotonergic neurons prepared from the embryonic rat brain. As shown in Fig. 5, 10 ng/ml of FGF-5 significantly (about 80%) increased the uptake of the neurotransmitter serotonin by cultured raphe neurons. The effects of the various neurotrophins on serotonin uptake in these cultures were smaller than those of FGF-5. Thus 10 ng per ml of either BDNF or NT-3 increased serotonin uptake only by 30% and 20% respectively, whereas NGF even seemed to have an inhibitory effect (see Fig. 5). The amount of serotonin uptake is thought to reflect the maturation of the serotonergic neurons (Foguet et al., 1993, EMBO J. 12(3):903-910). Therefore these results indicate that FGF-5 promotes the differentiation of the raphe serotonergic neurons to a greater extent than BDNF and NT-3.

6.3. DISCUSSION

6.3.1. REGULATION OF FGF-5 mRNA IN THE DEVELOPING RAT HIPPOCAMPUS

Here we show that, like NGF and BDNF-mRNAs (Berzaghi et al., 1993, J. Neurosci. 13:3818-3826), FGF-5 mRNA in the hippocampus is up-regulated by activation of muscarinic receptors in P7 rats. There is also a developmental increase in FGF-5 mRNA levels in the rat hippocampus parallelling that of NGF-mRNA (Auburger et al., 1987, Dev. Biol. 120:322-328). These findings together with the effect of scopolamine which itself lowered FGF-5 mRNA in the rat hippocampus strongly suggest that neuronal expression of FGF-5 mRNA in the developing rat hippocampus is controlled mainly by activation of muscarinic receptors. In favor of this, we observed in preliminary experiments that kainic acid, a non-NMDA receptor agonist, did not elevate FGF-5 mRNA in the rat hippocampus. Activation of kainic acid receptors has previously been shown to

induce the expression of BDNF and NGF-mRNA in the hippocampus of adult (Zafra et al., 1990, EMBO J. 9:3545-3550; Zafra et al., 1991, Proc. Natl. Acad. Sci. USA 88:10037-10041; Ballarin et al., 1991, Exp. Neurol. 114:35-43; Isackson et al., 1991, Neuron 6:937-948), but not in P7 rats (Dugich-Djordjevic et al., 1992, Neuron 8:1127-1138; Berzaghi et al., 1993, J. Neurosci. 13:3818-3826). Moreover, the fact that pilocarpine also increased the intensity of FGF-5 immunostaining (see below) in the rat hippocampus indicate that the increased levels of FGF-5 mRNA are translated into protein.

6.3.2. PRESENCE OF FGF-5 mRNA AND PROTEIN IN THE RAT HIPPOCAMPUS

In situ hybridization experiments and immunohistochemistry of brain sections showed the presence of FGF-5 mRNA and protein in the rat hippocampus. FGF-5 immunoreactivity was visualized by a specific peptide antiserum (Hughes et al., 1993, Neuron 10:369-377) and the staining was mainly localized in neurons of the dentate gyrus and the CA3 subregion. These subregions of the hippocampus also exhibited specific *in situ* hybridization signals for FGF-5 mRNA. The basal levels of FGF-5 immunoreactive material was low but could be increased by pilocarpine within 6 hours. Control experiments for FGF-5 immunohistochemistry included preabsorption of the antiserum with the FGF-5 peptide antigen which substantially decreased FGF-5 immunoreactivity in the rat hippocampus. A different peptide of the same length from the K-FGF, however, did not reduce the intensity of the FGF-5 staining.

The pattern of FGF-5 mRNA distribution in the rat hippocampus observed here is similar to that seen for FGF-5 mRNA in adult mouse brain employing *in situ*

hybridization (Haub et al., 1990, Proc. Natl. Acad. Sci. USA 87:8022-8026). Besides in hippocampus, we also observed specific hybridization for FGF-5 mRNA in a number of locations in the rat brain including rat cerebral cortex (Fig. 1). However, the exact cellular localization of FGF-5 mRNA in cortex, whether in neurons or non-neuronal cells, could not be determined conclusively. In addition, the presence of FGF-5 immunoreactive fiber elements in the rat hippocampus suggests that glial cells might produce FGF-5 as well.

However, like bFGF, FGF-5 might also be expressed by glial cells albeit at lower levels than in neurons.

6.3.3. FGF-5 PROMOTES DIFFERENTIATION OF CHOLINERGIC AND SEROTONERGIC NEURONS

Recombinant FGF-5 was found to enhance the differentiation of cultured septal cholinergic neurons and raphe serotonergic neurons. The effect of FGF-5 on these neurons occurred independently of the action of the neurotrophins. Thus, the effect of FGF-5 on the ChAT activity in septal cultures was not inhibited by anti-NGF-antibodies but was additive to that of NGF. We also observed that bFGF and FGF-5 had a similar capability in increasing ChAT activity in the septal neurons. Much higher concentration of bFGF (more than 100 ng per ml) have previously been shown to further enhance ChAT activity in cultured septal neurons (Knüsel et al., 1990, J. Neurosci. 10:558-570), possibly due to induction of some other growth factors by bFGF. Previous studies have shown that bFGF can increase the levels of NGF mRNA and NGF protein synthesized by cultured astrocytes (Spranger et al., 1990, Eur. J. Neurosci. 2:69-76; Yoshida and Gage, 1991, Brain Res. 538:118-126).

In contrast to septal cholinergic neurons, the effect of FGF-5 on serotonin uptake by the raphe

neurons was greater than that evoked by any of the neurotrophins tested (see Fig. 5). Recent studies have shown that cultured rat raphe neurons represent a good system for studying effects of different compounds on serotonergic neuron differentiation and neuronal gene expression (Foguet et al., 1993, EMBO J. 12(3):903-910). No reports on the growth factor requirements of serotonergic neurons are, however, yet available, but these neurons probably also require a trophic support from their targets. Fibers of serotonergic neurons in the raphe nucleus project rostrally to many brain areas including hippocampus (Lidov and Molliver, 1982, Brain Res. Bull. 8:389-430). Given the presence of FGF-5 in the rat hippocampus, FGF-5 may act as a target-derived trophic factor for the serotonergic neurons.

6.3.4. FUNCTIONAL IMPLICATIONS FOR FGF-5 IN THE RAT HIPPOCAMPUS

Taken together, our results show that FGF-5 enhances the differentiation of septal cholinergic and raphe serotonergic neurons. FGF-5 mRNA and FGF-5 protein are both expressed in the rat hippocampus which receives a dense cholinergic innervation from the septal region as well as serotonergic input from the rostral rhombencephalon. The effects of NGF and BDNF on septal cholinergic neurons are well-established and both factors increase ChAT activity in these neurons. As shown here, FGF-5 also increased ChAT activity in the cultured cholinergic neurons. FGF-5 mRNA in rat hippocampus also seems to be regulated in a similar manner during early postnatal development. Since FGF-5 contains a typical signal sequence it is probably also released in the rat hippocampus and thus might become available to the responsive neurons.

Various publications are cited herein that are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lindholm, Dan B.W.
Hartikka, Jukka A.
Berzaghi, Maria D.
Castren, Eero
Tzimagiorgis, Georgios
Hughes, Richard A.
Thoenen, Hans

(ii) TITLE OF INVENTION: Methods of Promoting the Survival and Differentiation of Subclasses of Cholinergic and Serotonergic Neurons Using Fibroblast Growth Factor-5

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: New York
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(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: Concurrently herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Misrock, S. Leslie
(B) REGISTRATION NUMBER: 18,872
(C) REFERENCE/DOCKET NUMBER: 8020-003-999

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 790-9090
(B) TELEFAX: (212) 790-8864/9741
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1123 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 140..943

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GACCCCCGCG GCTGGAAGA ATG AGC TTG TCC TTC CTC CTC CTC CTC TTC TTC       172
                        Met Ser Leu Ser Phe Leu Leu Leu Leu Phe Phe
                          1               5                  10

AGC CAC CTG ATC CTC AGC GCC TGG GCT CAC GGG GAG AAG CGT CTC GCC       220
Ser His Leu Ile Leu Ser Ala Trp Ala His Gly Glu Lys Arg Leu Ala
            15                  20                  25

CCC AAA GGG CAA CCC GGA CCC GCT GCC ACT GAT AGG AAC CCT ATA GGC       268
Pro Lys Gly Gln Pro Gly Pro Ala Ala Thr Asp Arg Asn Pro Ile Gly
        30                  35                  40

TCC AGC AGC AGA CAG AGC AGC AGT AGC GCT ATG TCT TCC TCT TCT GCC       316
Ser Ser Ser Arg Gln Ser Ser Ser Ser Ala Met Ser Ser Ser Ser Ala
    45                  50                  55

TCC TCC TCC CCC GCA GCT TCT CTG GGC AGC CAA GGA AGT GGC TTG GAG       364
Ser Ser Ser Pro Ala Ala Ser Leu Gly Ser Gln Gly Ser Gly Leu Glu
60                  65                  70                  75

CAG AGC AGT TTC CAG TGG AGC CCC TCG GGG CGC CGG ACC GGC AGC CTC       412
Gln Ser Ser Phe Gln Trp Ser Pro Ser Gly Arg Arg Thr Gly Ser Leu
                80                  85                  90

TAC TGC AGA GTG GGC ATC GGT TTC CAT CTG CAG ATC TAC CCG GAT GGC       460
Tyr Cys Arg Val Gly Ile Gly Phe His Leu Gln Ile Tyr Pro Asp Gly
            95                  100                 105

AAA GTC AAT GGA TCC CAC GAA GCC AAT ATG TTA AGT GTT TTG GAA ATA       508
Lys Val Asn Gly Ser His Glu Ala Asn Met Leu Ser Val Leu Glu Ile
        110                 115                 120

TTT GCT GTG TCT CAG GGG ATT GTA GGA ATA CGA GGA GTT TTC AGC AAC       556
Phe Ala Val Ser Gln Gly Ile Val Gly Ile Arg Gly Val Phe Ser Asn
    125                 130                 135

AAA TTT TTA GCG ATG TCA AAA AAA GGA AAA CTC CAT GCA AGT GCC AAG       604
Lys Phe Leu Ala Met Ser Lys Lys Gly Lys Leu His Ala Ser Ala Lys
140                 145                 150                 155

TTC ACA GAT GAC TGC AAG TTC AGG GAG CGT TTT CAA GAA AAT AGC TAT       652
Phe Thr Asp Asp Cys Lys Phe Arg Glu Arg Phe Gln Glu Asn Ser Tyr
                160                 165                 170

AAT ACC TAT GCC TCA GCA ATA CAT AGA ACT GAA AAA ACA GGG CGG GAG       700
Asn Thr Tyr Ala Ser Ala Ile His Arg Thr Glu Lys Thr Gly Arg Glu
            175                 180                 185

TGG TAT GTT GCC CTG AAT AAA AGA GGA AAA GCC AAA CGA GGG TGC AGC       748
Trp Tyr Val Ala Leu Asn Lys Arg Gly Lys Ala Lys Arg Gly Cys Ser
        190                 195                 200

CCC CGG GTT AAA CCC CAG CAT ATC TCT ACC CAT TTT CTT CCA AGA TTC       796
Pro Arg Val Lys Pro Gln His Ile Ser Thr His Phe Leu Pro Arg Phe
    205                 210                 215

AAG CAG TCG GAG CAG CCA GAA CTT TCT TTC ACG GTT ACT GTT CCT GAA       844
Lys Gln Ser Glu Gln Pro Glu Leu Ser Phe Thr Val Thr Val Pro Glu
220                 225                 230                 235

AAG AAA AAT CCA CCT AGC CCT ATC AAG TCA AAG ATT CCC CTT TCT GCA       892
Lys Lys Asn Pro Pro Ser Pro Ile Lys Ser Lys Ile Pro Leu Ser Ala
                240                 245                 250
```

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CCT CGG AAA AAT ACC AAC TCA GTG AAA TAC AGA CTC AAG TTT CGC TTT        940
Pro Arg Lys Asn Thr Asn Ser Val Lys Tyr Arg Leu Lys Phe Arg Phe
            255                 260                 265

GGA TAATATTAAT CTTGGCCTTG TGAGAAACCA TTCTTTCCCC TCAGGAGTTT             993
Gly

CTATAGGTGT CTTCAGAGTT CTGAAGAAAA ATTACTGGAC ACAGCTTCAG CTATACTTAC     1053

ACTGTATTGA AGTCACGTCA TTTGTTTCAG TGTGACTGAA ACAAAATGTT TTTTGATAGG     1113

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 268 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1               5                  10                  15

Ser Ala Trp Ala His Gly Glu Lys Arg Leu Ala Pro Lys Gly Gln Pro
            20                  25                  30

Gly Pro Ala Ala Thr Asp Arg Asn Pro Ile Gly Ser Ser Ser Arg Gln
        35                  40                  45

Ser Ser Ser Ser Ala Met Ser Ser Ser Ser Ala Ser Ser Ser Pro Ala
    50                  55                  60

Ala Ser Leu Gly Ser Gln Gly Ser Gly Leu Glu Gln Ser Ser Phe Gln
65                  70                  75                  80

Trp Ser Pro Ser Gly Arg Arg Thr Gly Ser Leu Tyr Cys Arg Val Gly
                85                  90                  95

Ile Gly Phe His Leu Gln Ile Tyr Pro Asp Gly Lys Val Asn Gly Ser
            100                 105                 110

His Glu Ala Asn Met Leu Ser Val Leu Glu Ile Phe Ala Val Ser Gln
        115                 120                 125

Gly Ile Val Gly Ile Arg Gly Val Phe Ser Asn Lys Phe Leu Ala Met
    130                 135                 140

Ser Lys Lys Gly Lys Leu His Ala Ser Ala Lys Phe Thr Asp Asp Cys
145                 150                 155                 160

Lys Phe Arg Glu Arg Phe Gln Glu Asn Ser Tyr Asn Thr Tyr Ala Ser
                165                 170                 175

Ala Ile His Arg Thr Glu Lys Thr Gly Arg Glu Trp Tyr Val Ala Leu
            180                 185                 190

Asn Lys Arg Gly Lys Ala Lys Arg Gly Cys Ser Pro Arg Val Lys Pro
        195                 200                 205

Gln His Ile Ser Thr His Phe Leu Pro Arg Phe Lys Gln Ser Glu Gln
    210                 215                 220
```

Pro Glu Leu Ser Phe Thr Val Thr Val Pro Glu Lys Lys Asn Pro Pro
225 230 235 240

Ser Pro Ile Lys Ser Lys Ile Pro Leu Ser Ala Pro Arg Lys Asn Thr
245 250 255

Asn Ser Val Lys Tyr Arg Leu Lys Phe Arg Phe Gly
260 265

WHAT IS CLAIMED IS:

1. A method of promoting differentiation of a septal cholinergic neuron comprising exposing said septal cholinergic neuron to an effective amount of fibroblast growth factor-5.

2. A method of promoting differentiation of a septal cholinergic neuron comprising exposing said septal cholinergic neuron to a concentration of fibroblast growth factor-5 which increases choline acetyltransferase activity in a sample of septal cholinergic neurons by at least 150 percent.

3. The method according to claim 1 in which said effective amount is a concentration of fibroblast growth factor-5 in the range of 1 nanogram per milliliter to 75 nanograms per milliliter.

4. A method of promoting differentiation of a septal cholinergic neuron comprising exposing said septal cholinergic neuron to an effective amount of a mixture of fibroblast growth factor-5 and a neurotrophin.

5. The method according to claim 4 in which the neurotrophin is nerve growth factor.

6. The method according to claim 4 in which the neurotrophin is brain-derived neurotrophic factor.

7. The method according to claim 4 in which the neurotrophin is neurotrophin-3.

8. The method according to claim 4 in which the neurotrophin is neurotrophin-4.

9. The method according to claim 4 in which said effective amount is a concentration of fibroblast growth factor-5 in the range of 1 nanogram per milliliter to 75 nanograms per milliliter and a concentration of neurotrophin in the range of 1 nanogram per milliliter to 50 nanograms per milliliter.

10. The method according to claim 4 in which said effective amount is a concentration of fibroblast growth factor-5 and a concentration of neurotrophin together being effective to increase choline acetyltransferase activity in a sample of septal cholinergic neurons by at least 150 percent.

11. A method of promoting differentiation of a raphe serotonergic neuron comprising exposing said raphe serotonergic neuron to an effective amount of fibroblast growth factor-5.

12. A method of promoting differentiation of a raphe serotonergic neuron comprising exposing said raphe serotonergic neuron to a concentration of fibroblast growth factor-5 which increases serotonin uptake by raphe serotonergic neurons by a sample of at least 50 percent.

13. The method according to claim 11 in which said effective amount is a concentration of fibroblast growth factor-5 in the range of 1 nanogram per milliliter to 100 nanograms per milliliter.

14. A method of promoting differentiation of a raphe serotonergic neuron comprising exposing said raphe serotonergic neuron to an effective amount of a mixture of fibroblast growth factor-5 and a neurotrophin.

15. The method according to claim 14 in which the neurotrophin is brain-derived neurotrophic factor.

16. The method according to claim 14 in which the neurotrophin is neurotrophin-3.

17. The method according to claim 14 in which said effective amount is a concentration of fibroblast growth factor-5 in the range of 1 nanogram per milliliter to 100 nanograms per milliliter and a concentration of neurotrophin is in the range of 1 nanogram per milliliter to 50 nanograms per milliliter.

18. The method according to claim 14 in which said effective amount is a concentration of fibroblast growth factor-5 and a concentration of neurotrophin together being effective to increase serotonin uptake in a sample of raphe serotonergic neurons by at least 50 percent.

19. A method of increasing the expression of fibroblast growth factor-5 in the hippocampus of a subject comprising administering, to the subject, an effective amount of a muscarinic receptor agonist.

20. A method of promoting or maintaining a neural circuit characterized by a neuron which has a fiber process that projects to the hippocampus,

comprising exposing the neuron to an effective amount of fibroblast growth factor-5.

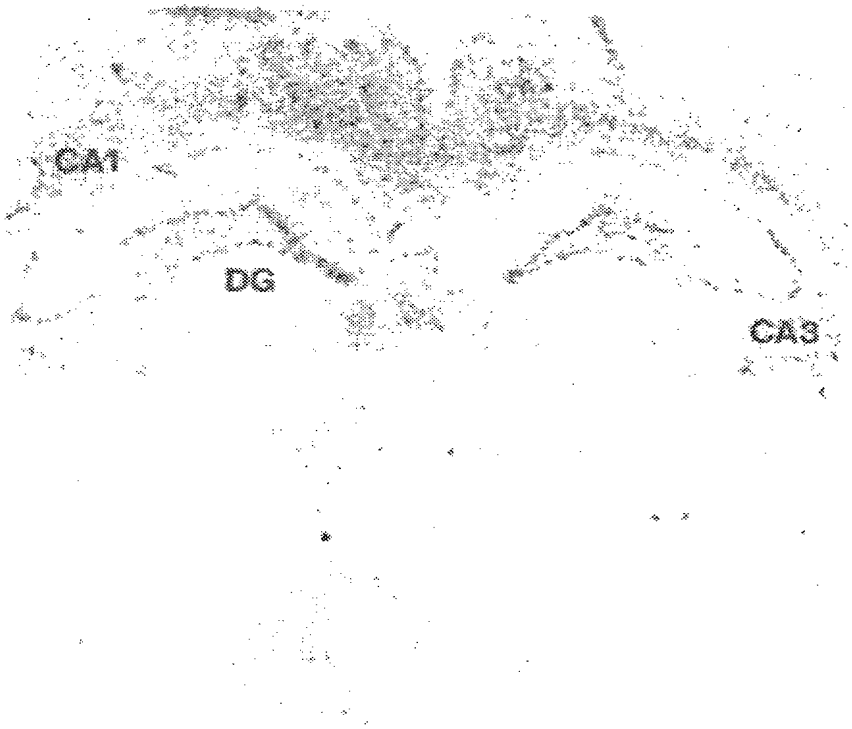

FIG. 1

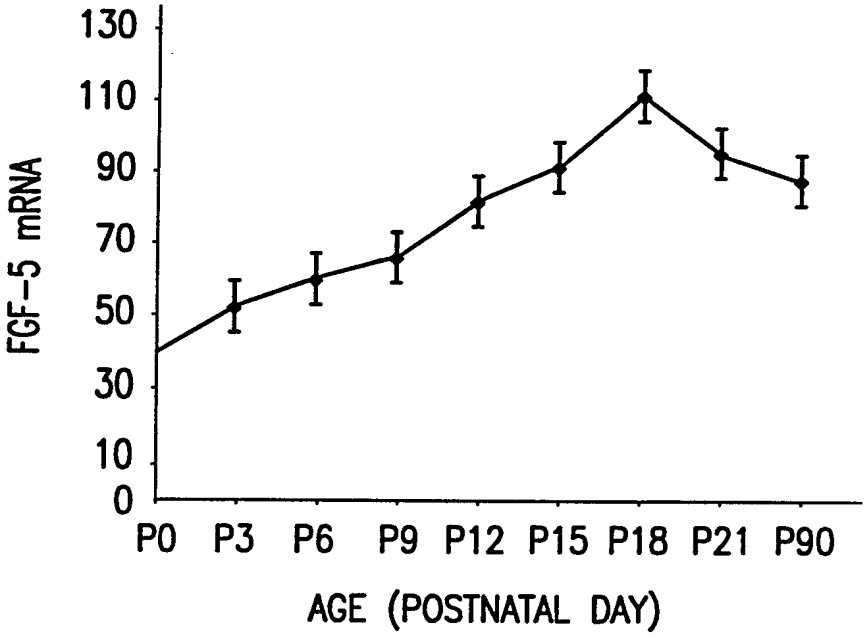
130
110
90
70
50
30
10
0
FGF-5 mRNA
P0 P3 P6 P9 P12 P15 P18 P21 P90
AGE (POSTNATAL DAY)

FIG.2A

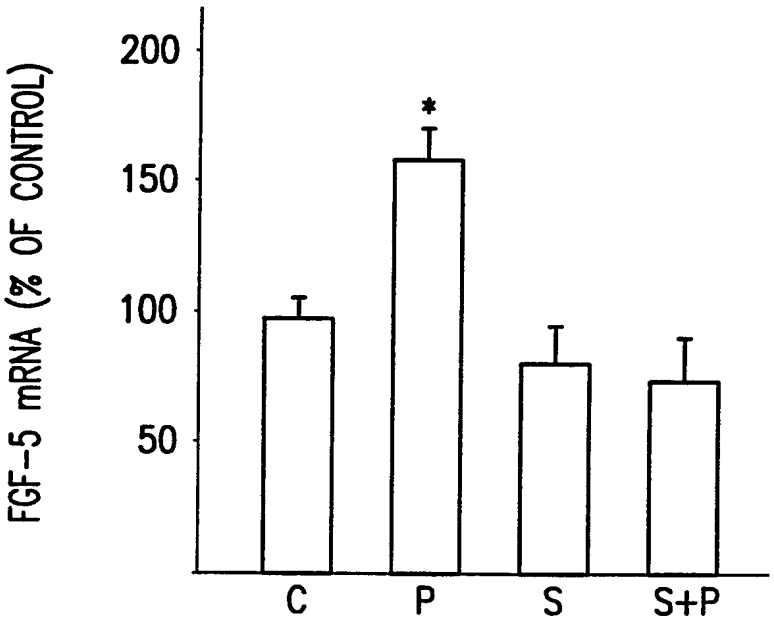
200
150
100
50
FGF-5 mRNA (% OF CONTROL)
*
C
P
S
S+P

FIG.2B

FIG.3A

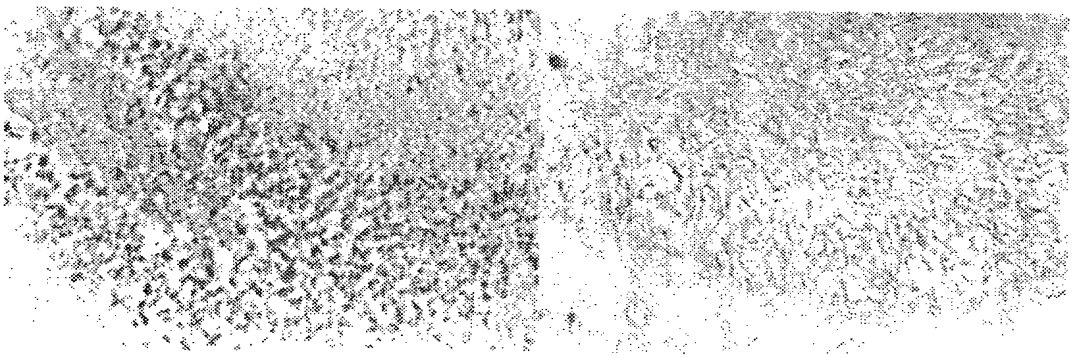

FIG.3B

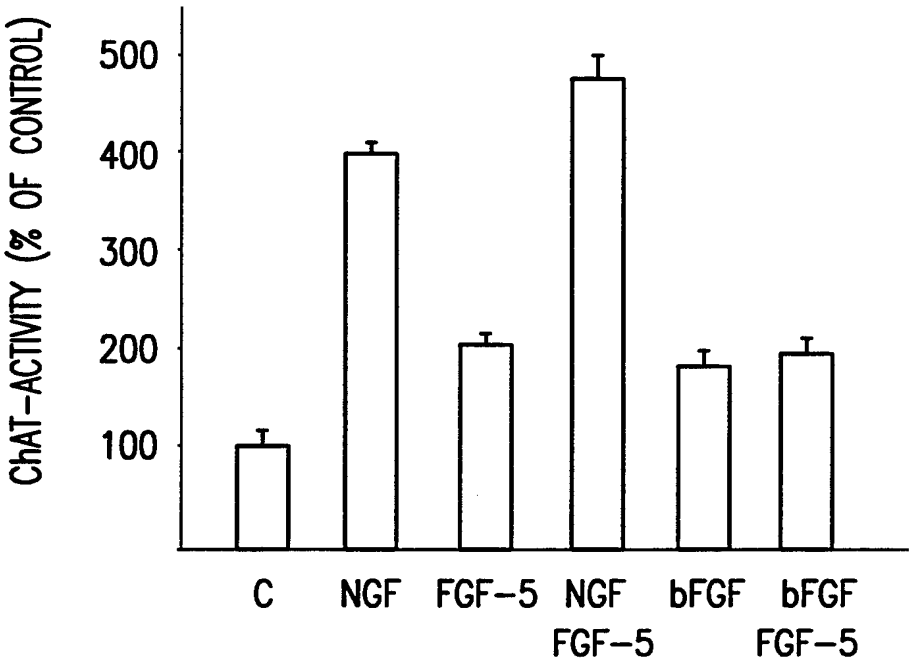
ChAT-ACTIVITY (% OF CONTROL)
500
400
300
200
100
C
NGF
FGF-5
NGF FGF-5
bFGF
bFGF FGF-5

FIG.4A

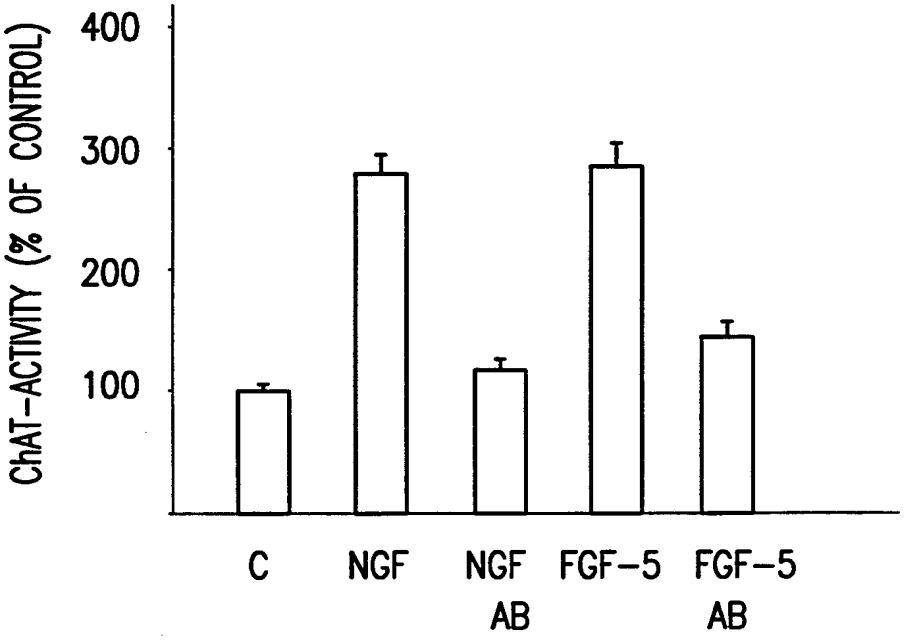
ChAT-ACTIVITY (% OF CONTROL)
400
300
200
100
C
NGF
NGF AB
FGF-5
FGF-5 AB

FIG.4B

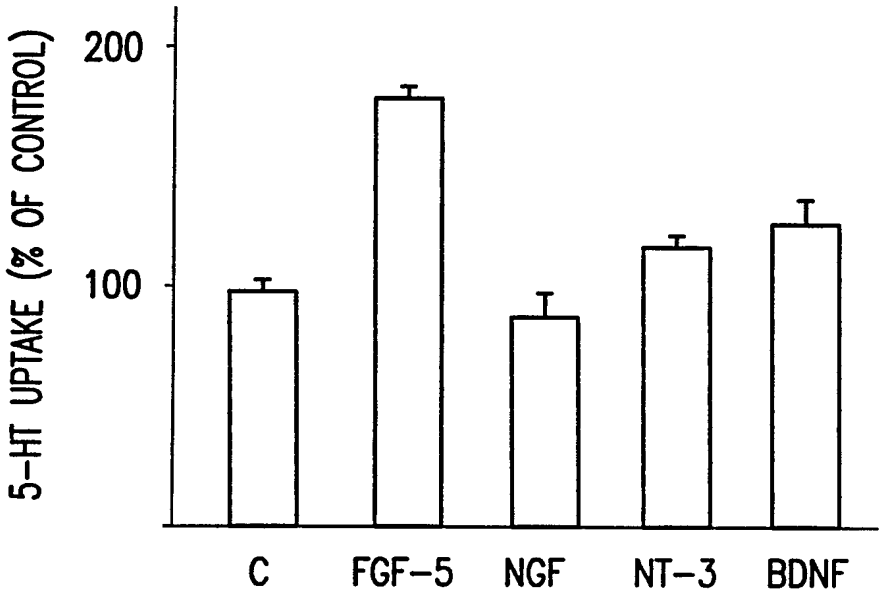

FIG.5

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GAGGCACGCA GCCGCACAGG GGCTACAGAG CCCAGAATCA GCCCTACAAG ATGCACTTAG     120

GACCCCCGCG GCTGGAAGA ATG AGC TTG TCC TTC CTC CTC CTC CTC TTC TTC       172
                     Met Ser Leu Ser Phe Leu Leu Leu Leu Phe Phe
                       1               5                  10

AGC CAC CTG ATC CTC AGC GCC TGG GCT CAC GGG GAG AAG CGT CTC GCC       220
Ser His Leu Ile Leu Ser Ala Trp Ala His Gly Glu Lys Arg Leu Ala
            15                  20                  25

CCC AAA GGG CAA CCC GGA CCC GCT GCC ACT GAT AGG AAC CCT ATA GGC       268
Pro Lys Gly Gln Pro Gly Pro Ala Ala Thr Asp Arg Asn Pro Ile Gly
        30                  35                  40

TCC AGC AGC AGA CAG AGC AGC AGT AGC GCT ATG TCT TCC TCT TCT GCC       316
Ser Ser Ser Arg Gln Ser Ser Ser Ser Ala Met Ser Ser Ser Ser Ala
    45                  50                  55

TCC TCC TCC CCC GCA GCT TCT CTG GGC AGC CAA GGA AGT GGC TTG GAG       364
Ser Ser Ser Pro Ala Ala Ser Leu Gly Ser Gln Gly Ser Gly Leu Glu
60                  65                  70                  75

CAG AGC AGT TTC CAG TGG AGC CCC TCG GGG CGC CGG ACC GGC AGC CTC       412
Gln Ser Ser Phe Gln Trp Ser Pro Ser Gly Arg Arg Thr Gly Ser Leu
                80                  85                  90

TAC TGC AGA GTG GGC ATC GGT TTC CAT CTG CAG ATC TAC CCG GAT GGC       460
Tyr Cys Arg Val Gly Ile Gly Phe His Leu Gln Ile Tyr Pro Asp Gly
            95                  100                 105

AAA GTC AAT GGA TCC CAC GAA GCC AAT ATG TTA AGT GTT TTG GAA ATG       508
Lys Val Asn Gly Ser His Glu Ala Asn Met Leu Ser Val Leu Glu Ile
        110                 115                 120

TTT GCT GTG TCT CAG GGG ATT GTA GGA ATA CGA GGA GTT TTC AGC AAC       556
Phe Ala Val Ser Gln Gly Ile Val Gly Ile Arg Gly Val Phe Ser Asn
    125                 130                 135
```

FIG.6A

```
AAA TTT TTA GCG ATG TCA AAA AAA GGA AAA CTC CAT GCA AGT GCC AAG      604
Lys Phe Leu Ala Met Ser Lys Lys Gly Lys Leu His Ala Ser Ala Lys
140                 145                 150                 155

TTC ACA GAT GAC TGC AAG TTC AGG GAG CGT TTT CAA GAA AAT AGC TAT      652
Phe Thr Asp Asp Cys Lys Phe Arg Glu Arg Phe Gln Glu Asn Ser Tyr
                160                 165                 170

AAT ACC TAT GCC TCA GCA ATA CAT AGA ACT GAA AAA ACA GGG CGG GAG      700
Asn Thr Tyr Ala Ser Ala Ile His Arg Thr Glu Lys Thr Gly Arg Glu
            175                 180                 185

TGG TAT GTT GCC CTG AAT AAA AGA GGA AAA GCC AAA CGA GGG TGC AGC      748
Trp Tyr Val Ala Leu Asn Lys Arg Gly Lys Ala Lys Arg Gly Cys Ser
        190                 195                 200

CCC CGG GTT AAA CCC CAG CAT ATC TCT ACC CAT TTT CTT CCA AGA TTC      796
Pro Arg Val Lys Pro Gln His Ile Ser Thr His Phe Leu Pro Arg Phe
    205                 210                 215

AAG CAG TCG GAG CAG CCA GAA CTT TCT TTC ACG GTT ACT GTT CCT GAA      844
Lys Gln Ser Glu Gln Pro Glu Leu Ser Phe Thr Val Thr Val Pro Glu
220                 225                 230                 235

AAG AAA AAT CCA CCT AGC CCT ATC AAG TCA AAG ATT CCC CTT TCT GCA      892
Lys Lys Asn Pro Pro Ser Pro Ile Lys Ser Lys Ile Pro Leu Ser Ala
                240                 245                 250

CCT CGG AAA AAT ACC AAC TCA GTG AAA TAC AGA CTC AAG TTT CGC TTT      940
Pro Arg Lys Asn Thr Asn Ser Val Lys Tyr Arg Leu Lys Phe Arg Phe
            255                 260                 265

GGA TAATATTAAT CTTGGCCTTG TGAGAAACCA TTCTTTCCCC TCAGGAGTTT           993
Gly

CTATAGGTGT CTTCAGAGTT CTGAAGAAAA ATTACTGGAC ACAGCTTCAG CTATACTTAC  1053

ACTGTATTGA AGTCACGTCA TTTGTTTCAG TGTGACTGAA ACAAAATGTT TTTTGATAGG  1113

AAGGAAACTG                                                         1123
```

FIG.6B

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/EP 94/03951

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEUROSCIENCE, vol.31, no.3, 1989 pages 649 - 661 GROTHE C. 'Basic fibroblast growth factor promotes in vitro survival and cholinergic development of rat septal neurons ..' see the whole document ---	1-20
X	NATURE, vol.332, no.6162, 24 March 1988, LONDON GB pages 360 - 361 ANDERSON K. 'Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo' cited in the application see the whole document --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 1995	03.03.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 08828 (SYNTEX-SYNERGEN NEUROSCIENCE JOINT VENTURE) 13 May 1993 see the whole document	1-20
A	NEURON, vol.10, no.3, March 1993 pages 369 - 377 HUGHES R.A. 'Evidence That Fibroblast Growth factor 5 Is a Major Muscle-Derived Survival Factor for Cultured Spinal Motoneurons' cited in the application see the whole document	1-20
P,X	THE EUROPEAN JOURNAL OF NEUROSCIENCE, vol.6, no.2, 1 February 1994 pages 244 - 252 LINDHOLM D. 'Fibroblast Growth Factor-5 Promotes Differentiation of Cultured Rat Septal Cholinergic Raphe Serotonergic Neurons ..' see the whole document	1-20
P,A	WO,A,94 20125 (MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSCHAFTEN) 15 September 1994 see the whole document	1-20

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INTERNATIONAL SEARCH REPORT

.rnational application No.
PCT/EP 94/03951

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-20 (as far as in vivo method are concerned) are directed to a method of treatment of the human/animal the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 94/03951

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9308828	13-05-93	AU-A- 3129793	07-06-93
WO-A-9420125	15-09-94	AU-B- 6284194	26-09-94